

MRID NO. 442219-01

DATA EVALUATION RECORD
FISH LIFE-CYCLE TOXICITY TEST
GUIDELINE 72-5

1. CHEMICAL: Tebufenozide PC Code No.: 129026
2. TEST MATERIAL: ¹⁴C-RH-5992 Technical Purity: 96.1%
3. CITATION:
Authors: J.E. Rhodes and T. Leak
Title: Full Life-Cycle Toxicity of RH-5992
Technical to the Fathead Minnow
(*Pimephales promelas*) Under Flow-Through
Conditions
Study Completion Date: December 10, 1996
Laboratory: ABC Laboratories, Inc., Columbia, MO
Laboratory Report ID: 42408
Sponsor: Rohm and Haas Company, Spring House, PA
MRID No.: 442219-01
DP Barcode: D237130; D237151; D237155
4. REVIEWED BY: Miachel Rexrode, M.S., Fisheries Biologist,
ERB IV, EFED.
Signature: *Miachel Rexrode* Date: 9-8-98
APPROVED BY: Mah Shamin, Chief, ERB IV.
Signature: Date:
5. Study Parameters:
Test Species: *Pimephales promelas*
Age of Test Organism: <24 hours old embryos
Test Duration: 219 days
Study Method: Flow-Through
Type of Concentrations: Mean measured
6. CONCLUSIONS: This study is not scientifically sound and does not fulfill the guideline requirements for a fish full life-cycle toxicity test using fathead minnows. Based on mean measured concentrations, the LOEC and NOEC for fathead minnows exposed to RH-5992 were not calculated.



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Results Synopsis:

Most Sensitive Endpoint: eggs/spawn

8. ADEQUACY OF THE STUDY:

A. Classification: ~~invalid~~ *Supplemental - but study must be repeated,*

B. Rationale: The number of eggs per spawn in all treatments appeared to be significantly different when compared to solvent control (Williams' Test, Appendix I). However, the pooling of control data (vehicle and dilution controls) is not appropriate in this case because of high variability in the dilution control (fig. 1). The data appears to represent a dose response which was confirmed through a regression analysis on the treatment means (Fig. 2). Because of the significant differences between vehicle control and treatments, the lowest observed effect concentration (LOEC) is 0.048 mg/L, but a value for the NOEC can not be calculated from this data. According to the Pesticide Registration Analysis Rejection Rate Analysis (EPA 738-R-94-035) a chronic study is considered invalid if a NOEC is not produced for reproductive or growth effects.

C. Repairability: N/A *This study must be repeated.*

9. GUIDELINE DEVIATIONS: Dissolved oxygen concentrations were less than 60% on several occasions. Diluter flow rate was increased to overcome the low DO concentrations.

10. SUBMISSION PURPOSE:

11. MATERIALS AND METHODS:

A. Biological System:

| Guideline Criteria | Reported Information |
|---|---|
| <p>Feeding: Fish should be fed at least twice daily and should not be fed for at least 24 hours prior to test termination.</p> | <p>Newly hatched fry were fed live rotifers (<i>Brachionus</i> sp.) and brine shrimp nauplii (<i>Artemia</i>). As the study progressed (Day 33), a commercial salmon starter was added to the diet. Salmon starter pellet size was increased over the course of the study. Food was provided <i>ad libitum</i> two to three times each day. Food was withheld 24 hours prior to each growth measurement and at study termination.</p> |
| <p>Embryo Exposure (Four-Five Days): Embryos (≤ 24 hours old) from at least 3 separate spawns should be randomly distributed to embryo cups.</p> <p>A minimum of 50 embryos (≤ 24 hrs old) per replicate cup, 4 cups per treatment should be used.</p> <p><u>Parameters measured:</u></p> <ul style="list-style-type: none"> • Survival of embryos • Time required to hatch • Hatching success • Survival of fry for 4 weeks <p>Dead and fungused embryos should be counted and removed daily.</p> | <p>Embryos (< 24 hours old) from 18 spawning tiles were randomly distributed to embryo cups.</p> <p>50 embryos per cup (randomly assigned five at a time); 1 cup per replicate test chamber; 4 replicate chambers per treatment and control.</p> <p>All parameters listed at left were measured.</p> <p>Dead embryos were recorded and removed daily until hatching was complete.</p> |

| Guideline Criteria | Reported Information |
|--|--|
| <p>Larval-Juvenile Exposure (From Hatch to 8 Weeks): After hatching, each group of larvae is randomly reduced to a minimum of 25 fish and released in replicate larval growth chambers. The random selection must include any fish that are lethargic or deformed.</p> <p><u>Parameters measured:</u></p> <ul style="list-style-type: none">• Fish survival (determined by counting the number of live fish in each replicate growth chamber weekly).• Total lengths (mm) of all fish at 4 and 8 weeks after hatching. | <p>95% hatch was complete on Day 5. Larvae from the incubation cups in each control and treatment chamber were impartially reduced to 25 on Day 7 and released into the larval growth chamber on Day 9. At 56 days post-hatch, juveniles were impartially reduced to 20 per replicate.</p> <p>Both parameters at left were measured.</p> |

| Guideline Criteria | Reported Information |
|---|---|
| <p>Juvenile-Adult Exposure (From 8 wks posthatch to the end of the spawning phase [32-40 wks]):</p> <p>At 20-24 weeks after hatching, mature fish are placed in a spawning tank of the same concentration (4 males and 4 females randomly chosen and assigned). The spawning tank is divided into 4 individual spawning chambers with appropriate spawning substrates.</p> <p>The substrates are examined daily and embryos removed, counted, and recorded separately for each pair.</p> <p>For fathead minnow, adult exposure should be terminated when no spawning occurs for one week.</p> <p>For sheepshead minnow, testing should be terminated after spawning is observed for 2 weeks.</p> | <p>Each test aquarium was divided into 4 equal spawning compartments. On Day 93, one male and one female were distributed to each spawning compartment. The remaining adults were sacrificed on Day 106. Spawning substrate consisted of U-shaped sandblasted stainless steel tile.</p> <p>Substrates were examined, and embryos were removed and counted daily.</p> <p>The spawning phase was terminated at 182 days post-hatch.</p> |
| <p>Second Generation Embryo Exposure (4-5 days):</p> <p>50 embryos from each conc. level are randomly selected and transferred to incubation cups for hatch. Use the same test procedures as those for parental generation.</p> <p>Embryos not selected are discarded.</p> | <p>4-8 sets of 35 embryos from each replicate were incubated and the percent hatch was determined.</p> <p>Not reported.</p> |

| Guideline Criteria | Reported Information |
|--|--|
| <p>Second Generation Larval-Juvenile Exposure (From Hatch to 4-8 wks): After hatching, 25 larvae are released in each growth chambers (2 chambers per treatment).</p> <p>Each group of 2nd generation fish is terminated 8 wks after hatching.</p> <p>Fish are blotted, weighed, and measured before being discarded.</p> | <p>3-4 groups of 21-25 larvae were released into growth chambers for each treatment.</p> <p>After 8 wks of post-hatch exposure the 2nd generation fish were terminated.</p> <p>Fish were measured for individual length and wet weight.</p> |

Comments: None.

B. Physical System:

| Guideline Criteria | Reported Information |
|--|---|
| <p>Test Water: <u>Sheepshead Minnow</u> 1. Natural seawater (sterilized and filtered) or a commercial mixture. 2. Natural seawater with a salinity of ≥ 15 parts per thousand (weekly range of salinity $< 6\%$ and monthly pH range < 0.8 pH units).</p> <p><u>Fathead Minnow</u> 1. Reconstituted water or water from unpolluted well or spring (sterilized and tested for pollutants). 2. Hardness of 40 to 48 mg/L as CaCO_3 and pH of 7.2 to 7.6.</p> | <p>1. Well water, filtered through reverse osmosis, tested, heated and passed through a polypropylene cartridge filter containing an ultraviolet sterilizer before delivery to the proportional diluter.</p> <p>2. The hardness ranged from 128 to 150 mg/L as CaCO_3. The pH ranged from 7.60 to 8.44.</p> |

| Guideline Criteria | Reported Information |
|---|--|
| Test Temperature: <u>Fathead:</u> 25°C and should not remain outside the range of 24 to 26°C for more than 48 hours. <u>Sheepshead:</u> 30°C. | Range: 24.3 - 26.0°C |
| Photoperiod: 16-hour light/8-hour dark. Light intensity of 10-100 lumens at water surface. | Varying light:dark photoperiod designed to simulate the national average (Evansville, IN). Within the range of 57 - 83 lumens at the water surface. |
| Dosing Apparatus: 1. Intermittent flow proportional diluters or continuous flow serial diluters. 2. A minimum of 5 toxicant concentrations with a dilution factor ≤ 0.5 . 3. One control should be used. | 1. Intermittent flow proportional diluter. 2. Five with a dilution factor of 0.5. 3. A dilution water control and a solvent control. |
| Toxicant Mixing: 1. Mixing chamber recommended but not required. 2. Test solution completely mixed before introduction into the test system (aeration should not be used for mixing). 3. Flow splitting accuracy must be within 10% and periodically checked. | 1. Mixing chambers were used 2. Not reported. 3. According to ABC protocol, flow splitting accuracy was calibrated to within 10% and checked periodically. |

| Guideline Criteria | Reported Information |
|--|--|
| <p>Exposure System/Test Vessels: Exposure tanks should be all glass or glass with a plastic or stainless steel frame (30.5 x 30.5 x 91.4 cm or 30.5 x 30.5 x 61 cm for fathead, and 45 x 90 x 26 cm for sheephead).</p> <p>Larval chambers should have glass bottoms and drains that allow water to be drawn down to 3 cm.</p> <p>Test water depth in adult tanks and larval chambers should be a minimum of 15 cm.</p> | <p>Glass exposure tanks were divided into two chambers with a glass partition. Each chamber measured 20.2 X 19 X 33 cm with a test solution volume of 9.8 L. Each duplicate spawning aquarium measured approximately 52 X 36.9 X 33 cm, filled to a volume of approximately 45 L.</p> <p>26.5 cm depth within growth tanks; 23.4 cm depth within spawning chambers.</p> |
| <p>Embryo and Fry Chambers: 120 ml glass jars with bottoms replaced with 40 mesh stainless steel or nylon screen. Chambers can be oscillated vertically using rocker arm apparatus (2 rpm motor) or placed in separate chambers with self-starting siphons.</p> | <p>Incubation chambers were 9-cm diameter flint glass jars with 40-mesh Nitex® screen bottoms. Incubation cups were suspended within each replicate growth chamber and oscillated vertically by means of a rocker arm apparatus driven by a low rpm electric motor.</p> |
| <p>Flow Rate: Flow rates to larval cups should provide 90% replacement in 8-12 hours, and maintain DO at above 75% of saturation. The toxicant level cannot drop below 20% with fish in the tank.</p> | <p>Flow rate to exposure tanks during the growth phase (Days 0-89) was 13 vol/24 hours. Flow rate to spawning tanks (Days 89-219) was 11 vol/24 hours.</p> <p>DO levels ranged from 3.0 - 8.1 mg/L (38-103% saturation); mean DO levels levels ranged from 67 to 81% saturation.</p> <p>Toxicant levels remained constant throughout the duration of the study; mean recoveries were 92-100% of nominal.</p> |

| Guideline Criteria | Reported Information |
|---|---|
| Aeration: Dilution water should be aerated to insure DO concentration at or near 100% saturation. Test tanks and embryo chambers should not be aerated. | Pure oxygen was injected into the incoming water cell on the diluter face beginning on Day 166. Tank aeration was conducted only on Days 182-183. |

Comments: Early on Day 182, a diluter malfunction prevented flow of dilution water and test solutions to the exposure aquaria for approximately 12-14 hours, resulting in unacceptable DO concentrations and scattered mortality of sexually mature F_0 adults and F_1 juveniles. The exposure tanks were aerated and the diluter repaired by the afternoon of the same day.

C. Chemical System:

| Guideline Criteria | Reported Information |
|---|---|
| Concentrations: Minimum of 5 concentrations and a control, all replicated; plus solvent control if appropriate. Toxicant conc. must be measured in one tank at each toxicant level every week. | A dilution water control, solvent control and 5 treatment concentrations: 0.048, 0.095, 0.19, 0.38, and 0.75 mg ai/L. Test solutions were sampled and measured once a week from each replicate aquarium of the controls and treatments (replicates were composited). During the reproductive and F_1 phases, samples were taken from the incubation chambers or diluter taps prior to entering test aquaria. |

| Guideline Criteria | Reported Information |
|--|--|
| <p>Other Variables:</p> <ol style="list-style-type: none"> 1. DO must be measured at each conc. at least once a week. 2. Test water temp. must be recorded continuously. 3. <u>Freshwater</u>: A control and one conc. must be analyzed weekly for pH, alkalinity, hardness, and conductance. <u>Natural seawater</u>: must maintain a constant salinity and not fluctuate more than 6% weekly; monthly pH range <0.8 pH units. | <ol style="list-style-type: none"> 1. DO and temperature were measured on Days 0, 14, and at least weekly thereafter in two of the four replicate growth chambers or in at least one partitioned area of both spawning aquaria of each control and treatment level. 2. Temperature was measured twice daily and continuously in a "centrally located chamber" in addition to the weekly measurements described above. 3. Conductivity, hardness, and alkalinity were measured weekly in one replicate of the negative control, low, and high test concentrations. |
| <p>Solvents: Should not exceed 0.1 ml/L in a flow-through system. Acceptable solvents are: dimethylformamide, triethylene glycol, methanol, acetone, ethanol.</p> | <p>Solvent conc.: 0.02 mL/L Solvent: acetone</p> |

F₀ Results:

| Nominal Conc. (mg ai/L) | Mean Measured Conc. (mg ai/L) | % Hatch | Day 29 (4 wks post-hatch) % Survival | Day 56 (8 wks post-hatch) % Survival | Day 84* % Survival |
|----------------------------|----------------------------------|---------|--|--|-----------------------|
| Control | <0.50 | 87 | 94 | 92 | 100 |
| Solvent Control | <0.50 | 93 | 96 | 96 | 100 |
| 0.048 | 0.048 | 89 | 95 | 95 | 100 |
| 0.095 | 0.090 | 87 | 97 | 96 | 100 |
| 0.19 | 0.18 | 89 | 90 | 90 | 99 |
| 0.38 | 0.35 | 88 | 66** | 66** | 71** |
| 0.75 | 0.72 | 94 | 33** | 33** | 31** |

* Fish were impartially thinned to 20/replicate after Day 56 with the exception of the 0.72 mg ai/L treatment (25/replicate).

** Statistically different from the solvent control.

| Mean Measured Concentration (mg ai/L) | Day 29 Length (mm) | Day 56 Length (mm) | Day 84 Length (mm) | Day 84 Wet Weight (g) |
|--|-----------------------|-----------------------|-----------------------|--------------------------|
| Control | 21.4 | 35.6 | 42.9 | 1.788 |
| Solvent Control | 21.6 | 36.1 | 43.3 | 1.820 |
| 0.048 | 21.7 | 36.0 | 43.6 | 1.832 |
| 0.090 | 21.4 | 35.4 | 42.8 | 1.772 |
| 0.18 | 21.6 | 35.9 | 43.3 | 1.821 |
| 0.35 | 22.2 | 36.8 | 43.1 | 1.755 |
| 0.72 | 21.8 | 35.8 | 43.3 | 1.755 |

| Mean Measured Conc. (mg ai/L) | Days to first Spawn | Total Number of Eggs | Number Eggs/Spawn |
|----------------------------------|---------------------|----------------------|-------------------|
| Control | 102 | 1672 | 105 |
| Solvent Control | 102 | 2098 | 168 |
| 0.048 | 134 | 1279 | 91* |
| 0.090 | 109 | 1211 | 120* |
| 0.18 | 110 | 1390 | 117* |
| 0.35 | 105 | 1594 | 113* |
| 0.72 | 129* | 599 | 67* |

462
292

* Significantly different from the solvent control.

F₁ Results:

| Mean Measured Concentration (mg ai/L) | % Hatch | 51-57 day Post-Hatch % Survival | 51-57 day Post-Hatch Length (mm) | 51-57 day Post-Hatch Wet Weight (g) |
|--|---------|---------------------------------|----------------------------------|-------------------------------------|
| Control | 90 | 90 | 36.3 | 0.938 |
| Solvent Control | 90 | 93 | 36.2 | 0.859 |
| 0.048 | 96 | 94 | 35.0 | 0.805 |
| 0.090 | 83 | 88 | 35.0 | 0.826 |
| 0.18 | 86 | 83 | 36.4 | 1.020 |
| 0.35 | 89 | 91 | 35.2 | 0.819 |
| 0.72 | 93 | 92 | 33.1* | 0.666* |

* Statistically different than the solvent control.

Toxicity Observations: Physical and behavioral abnormalities noted in the F₀ fish included spinal curvature, fish resting on the bottom of the test chamber, and loss of equilibrium. Spinal curvature was observed in two individuals in the solvent control, two fish in the 0.18 ppm ai treatment, and one fish in the 0.72 ppm ai treatment. The authors stated that "this response was not the result of exposure to RH-5992 and is not biologically significant." During the spawning phase, two females in the control, one in the solvent control, and one each in the 0.048, 0.35, and 0.72 ppm ai treatments exhibited abdominal bloating and subsequently died. The authors stated that this condition "was likely the result of being unable to resorb eggs from the ovaries." No physical and/or behavioral abnormalities were noted in F₁ generation fish during the study.

Comments: None.

Statistical Results:

Statistical Method: Fisher's Exact Test or Student's t-test were used for continuous data (length and weight) and Dunnett's Test was used for survival and hatching success. All treatment data were compared to those of the pooled control.

| Biological Endpoint | NOEC (mg ai/L) | LOEC (mg ai/L) |
|------------------------------------|----------------|----------------|
| F ₀ hatching success | 0.72 | >0.72 |
| F ₀ 29-day survival | 0.18 | 0.35 |
| F ₀ 29-day length | 0.72 | >0.72 |
| F ₀ 56-day survival | 0.18 | 0.35 |
| F ₀ 56-day length | 0.72 | >0.72 |
| F ₀ 84-day survival | 0.18 | 0.35 |
| F ₀ 84-day length | 0.72 | >0.72 |
| F ₀ 84-day wet weight | 0.72 | >0.72 |
| F ₀ days to first spawn | 0.72 | >0.72 |
| F ₀ eggs/spawn | - | 0.048 |
| F ₁ hatching success | 0.72 | >0.72 |
| F ₁ 51-57 day survival | 0.72 | >0.72 |
| F ₁ 51-57 day length | 0.35 | 0.72 |
| F ₁ 51-57 day weight | 0.35 | 0.72 |

NOEC: - LOEC: 0.048 mg ai/L

Comments: None.

13. REVIEWER'S STATISTICAL RESULTS:

Statistical Method: Bonferroni's Test and Dunnett's Test were used for continuous data (length and weight) and Williams' Test was used for survival and hatching success. All comparisons were made against the solvent control.

| Biological Endpoint | NOEC (mg ai/L) | LOEC (mg ai/L) |
|-------------------------------------|----------------|----------------|
| F ₀ hatching success | 0.72 | >0.72 |
| F ₀ 29-day survival | 0.18 | 0.35 |
| F ₀ 29-day length | 0.72 | >0.72 |
| F ₀ 56-day survival | 0.18 | 0.35 |
| F ₀ 56-day length | 0.72 | >0.72 |
| F ₀ 84-day survival | 0.18 | 0.35 |
| F ₀ 84-day length | 0.72 | >0.72 |
| F ₀ 84-day wet weight | 0.72 | >0.72 |
| F ₀ days to first spawn | 0.35 | 0.72 |
| F ₀ eggs/spawn | - | 0.048 |
| F ₁ hatching success | 0.72 | >0.72 |
| F ₁ 51-57 day survival | 0.72 | >0.72 |
| F ₁ 51-57 day length | 0.35 | 0.72 |
| F ₁ 51-57 day wet weight | 0.35 | 0.72 |

Most sensitive endpoint(s): eggs/spawn

NOEC: - LOEC: 0.048 mg ai/L

Comments: The number of eggs/spawn in all treatments were significantly less than the solvent control. The reviewer believes that this result is treatment-related and that a dose-response relationship appears to be occurring. The use of pooled controls is not appropriate because of high variability in the dilution control. The NOEC was not calculated.

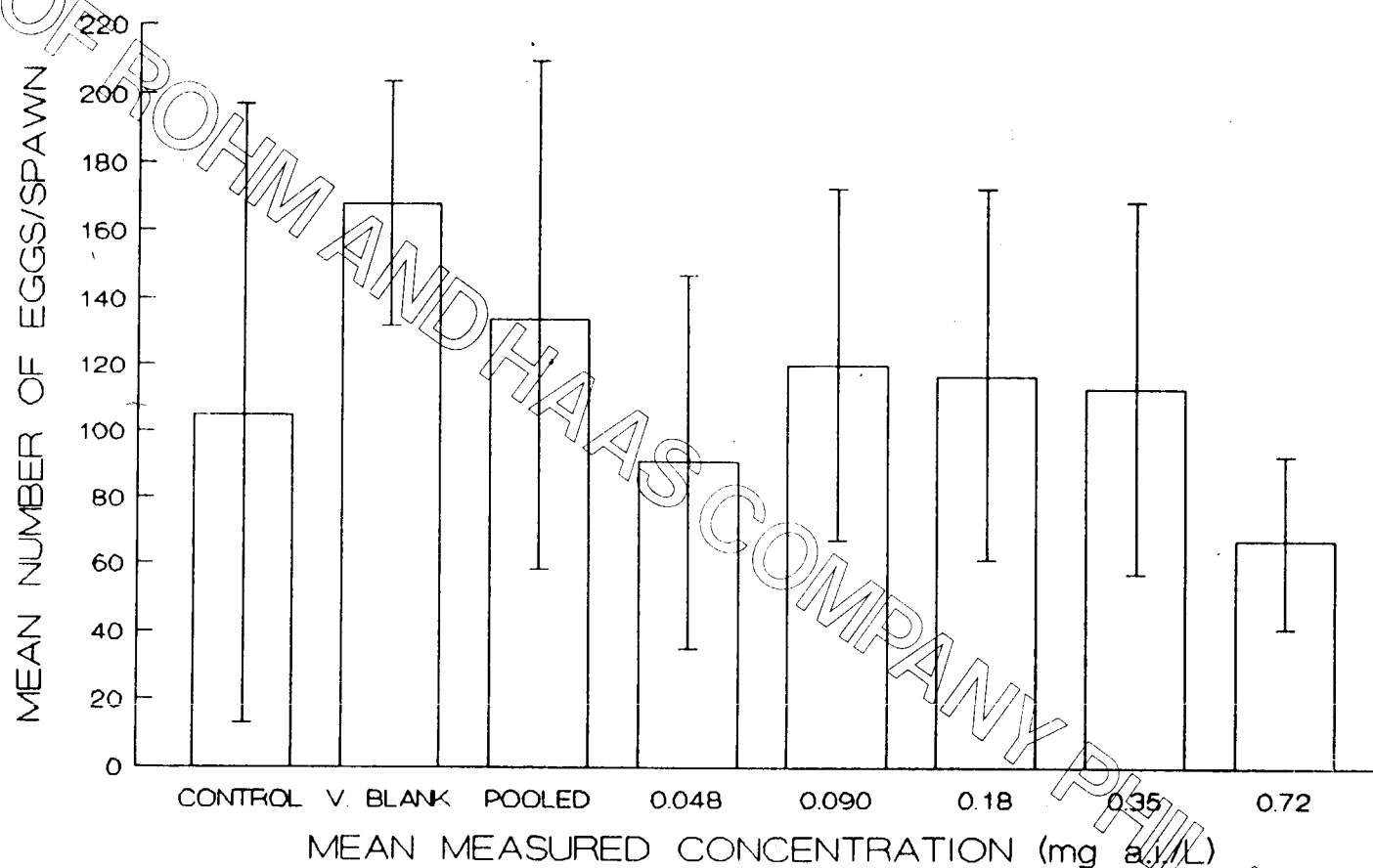
14. REVIEWER'S COMMENTS:

This study does not appear to be scientifically sound and does not fulfill the guideline requirements for a fish full life-cycle toxicity test using fathead minnows. Given the endocrine disruptor effects of this compound in other organisms, this reviewer is concerned about related effects in fish reproduction. Possible credence is given for this argument when it is noted that there appeared to be significant differences between vehicle control and treatments when evaluating number of eggs per spawn. Using this information, the lowest observed effect concentration (LOEC) is 0.048 mg/L, but a value for the NOEC can not be calculated from this data. According to the Pesticide Registration Analysis Rejection Rate Analysis (EPA 738-R-94-035) a chronic study is considered invalid if a NOEC is not produced for reproductive or growth effects. Therefore, this study is classified as invalid.

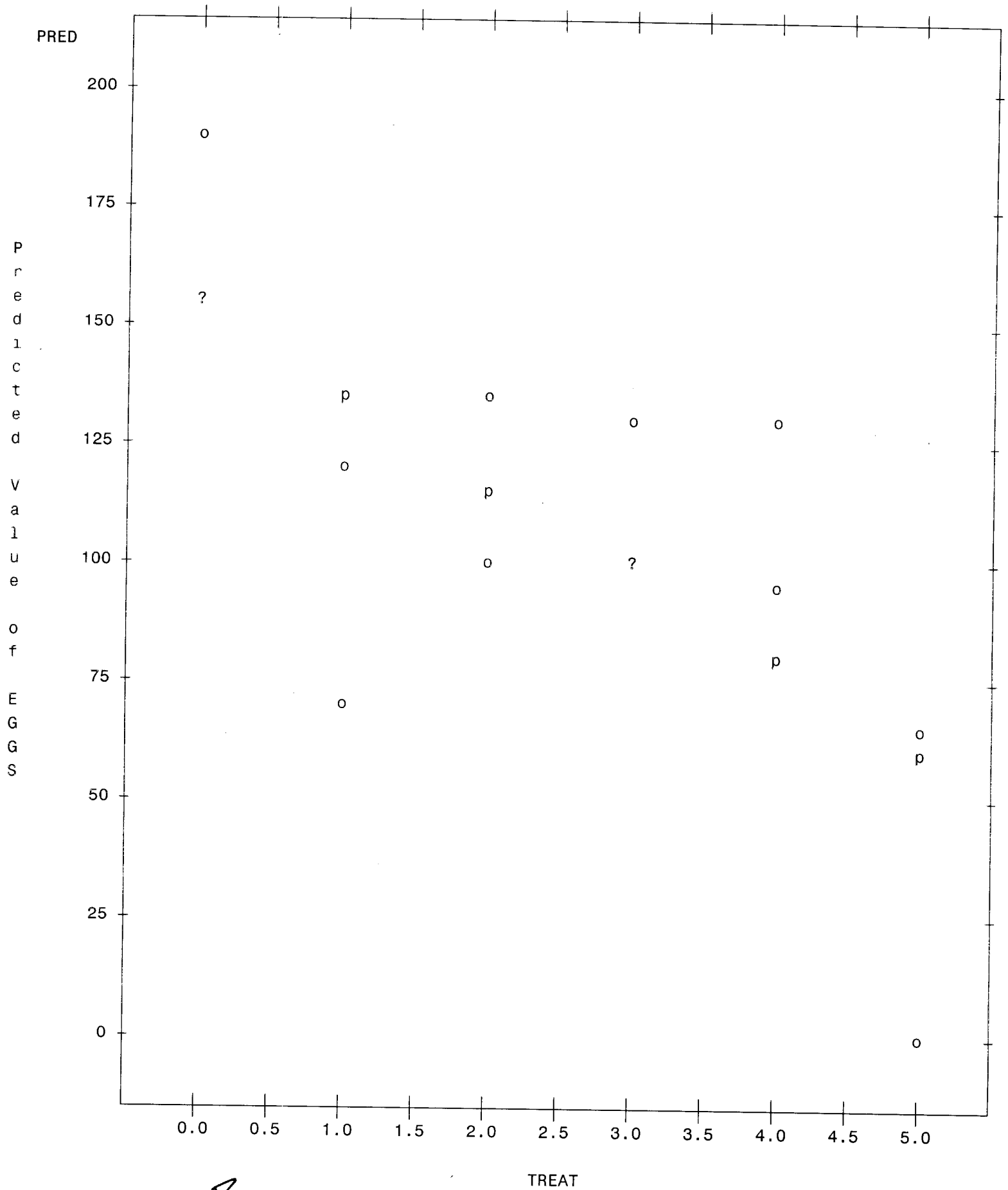
Five issues must be considered in this test: 1) The number of eggs per spawn in all treatments appeared to be significantly different when compared to solvent control (Williams' Test, Appendix I); 2) The pooling of control data (vehicle and dilution controls) is not appropriate in this case because of high variability in the dilution control (fig. 1); 3) The data appear to represent a dose response which was confirmed through a regression analysis of the treatment means (Fig. 2); 4) Mechanical problems. Dissolved oxygen concentrations were less than 60% on several occasions. On day 182, a malfunction of the dilute system timing device was discovered. This problem prevented flow of dilution water and test solutions to the exposure chambers for about 12-14 hours. As a result, the DO concentrations fell to unacceptable levels resulting in scattered mortality of sexually mature adults and F₁ generation juveniles; 5) this compound is an effector of an endocrine system in crustaceans, is there a conserved evolutionary connection in fish ?

FIGURE 1

Mean Number of Eggs/Spawn Exhibited by Fathead Minnows
(*Pimephales promelas*) During Full Life-Cycle Exposure to RH-5992 Technical



No statistically significant ($P > 0.05$) reductions from pooled control group
Error bars represent the pooled standard deviation



Regression of Treatment Means

Appendix I Comparative Evaluation of Treatments to Vehicle Control using The Williams' Test

fathead full life

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WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

| GROUP | IDENTIFICATION | | ORIGINAL N | MEAN | TRANSFORMED MEAN | ISOTONIZED MEAN |
|-------|----------------|---|---------------|---------|---------------------|--------------------|
| 1 | veh control | 2 | 172.000 | 172.000 | 172.000 | |
| 2 | dil control | 2 | 113.000 | 113.000 | 113.000 | |
| 3 | 0.048 | 2 | 95.000 | 95.000 | 110.375 | |
| 4 | 0.090 | 2 | 117.000 | 117.000 | 110.375 | |
| 5 | 0.18 | 2 | 117.000 | 117.000 | 110.375 | |
| 6 | 0.35 | 2 | 112.500 | 112.500 | 110.375 | |
| 7 | 0.72 | 2 | 33.500 | 33.500 | 33.500 | |

fathead full life

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WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

| IDENTIFICATION | ISOTONIZED MEAN | CALC. | SIG WILLIAMS | TABLE P=.05 | DEGREES OF WILLIAMS | FREEDOM |
|----------------|--------------------|-------|-----------------|----------------|------------------------|---------|
| veh control | 172.000 | | | | | |
| dil control | 113.000 | 1.957 | * | 1.89 | k= 1, v= 7 | |
| 0.048 | 110.375 | 2.044 | * | 2.00 | k= 2, v= 7 | |
| 0.090 | 110.375 | 2.044 | * | 2.04 | k= 3, v= 7 | |
| 0.18 | 110.375 | 2.044 | | 2.06 | k= 4, v= 7 | |
| 0.35 | 110.375 | 2.044 | | 2.07 | k= 5, v= 7 | |
| 0.72 | 33.500 | 4.594 | * | 2.08 | k= 6, v= 7 | |

s = 30.145

Note: df used for table values are approximate when v > 20.